Title: Evaluation of the analytic variability of urine protein-to-creatinine ratio in cats

Analytical variability of feline proteinuria

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Abstract

Background: Quantification of proteinuria with urinary protein-to-creatinine (UPC) ratio is part of the diagnostic process in feline patients suspected of chronic kidney disease (CKD). In affected cats, monitoring and substaging of UPC according to International Renal Interest Society (IRIS) guidelines is also necessary for the appropriate patients' management. No information is available about the possible effect of analytical variability on urinary protein (UP) and UPC ratio in cats.

Objectives: The aim of this study was to determine whether imprecision and method-dependent difference due to the two dye-binding methods pyrogallol red-molybdate (PRM) and coomassie brilliant blue (CBB) could affect substaging according to IRIS guidelines.

Methods: Urine samples were collected from proteinuric and non-proteinuric cats. Intra-assay and inter-assay repeatability were assessed with both PRM and CBB. Urinary supernatants (n=120) were tested with both methods. Agreement between methods and concordance in samples classification according to IRIS guidelines were determined.

Results: On average, PRM yielded higher CV (UP: 8.4±5.2%; UPC: 9.5±4.8%) than CBB (UP: 5.6±2.6%; UPC: 7.2±2.6%) but similar rate of misclassifications were found in samples with UPC close to the IRIS cutoff. Although the two methods were correlated, CBB tended to yield UP and UPC values significantly higher ($P<0.0001$) than PRM. Constant and proportional errors between PRM and CBB were also found by the Passing Bablok test. Concordance in substaging samples according to IRIS was good (k coefficient =0.62).

Conclusion: The two methods were precise but the higher UPC obtained with CBB may affect interpretation of the IRIS guidelines and clinical decisions.

Keywords: Chronic Kidney Disease, Coomassie brilliant blue, International Renal Interest Society, Proteinuria, Pyrogallol Red, Urinalysis

Introduction

Chronic kidney disease (CKD) is the most common renal disease in cats and is defined as structural and/or functional impairment of one or both kidneys that has been present for more than 3 months.[1] CKD may result from heterogeneous causes, often not identified but that can induce a progressive and irreversible damage to the kidneys.[2]

Proteinuria is a sign of kidney damage, but also a strong indicator for progression of CKD.[3-5] It was hypothesized that proteinuria accelerates progression of CKD by direct toxic effect of reabsorbed proteins on tubular epithelial cells;[6] this chronic injury induces the release of cytokines, cellular apoptosis and tubular degeneration and atrophy, that, in turn, leads to interstitial inflammation and fibrosis.[7,8] Proteinuria in cats with naturally occurring CKD is generally mild, with 90% and 49% of cats with CKD having a UPC of <1.0 and <0.25, respectively.[9] The severity of proteinuria, however, has prognostic significance in terms of survival time.[9,10] Consequently, the ACVIM consensus statement on the treatment of proteinuria recommends therapeutic intervention when UPC ≥0.4 in cats with CKD causing azotaemia.[3]

Proteinuria can be routinely assessed via semi-quantitative methods, such as urine dipstick colorimetric test. However, false-positive reactions for proteins in healthy cats as well as in cats with CKD limit its utility.[4,11,12] A large amount of cauxin (a 70kDa glycoprotein) has been demonstrated in feline urine and it is responsible for false positive protein results on urine dipstick tests.[13] Therefore the single best test for the detection of proteinuria in cats is the UPC ratio.[14]

The International Interest Renal Society (IRIS) proposed sub-staging of feline CKD based on UPC ratio and defined non-proteinuric (NP) patients with UPC ratio ≤ 0.20, borderline proteinuric (BP) patients with UPC ratio from 0.21 to 0.40 and proteinuric (P) patients with UPC ratio >0.40.[15]

Although the gold standard for detection of proteinuria is the quantification of protein in a 24 hours urine collection, in feline medicine this approach is impractical in clinical settings. Currently, the quantification of proteinuria with the urinary protein-to-creatinine (UPC) ratio in spot urine sample is considered a reliable estimation of the daily protein excretion in cats.[16,17]

Although proteinuria in cats is routinely assessed as part of the diagnostic process in patients suspected of CKD,[1,3,15] to the authors' knowledge there is no information available about analytical factors that may affect the measurement of proteinuria. Dye-binding methods are easy to use, relatively rapid and inexpensive and there are several assays available to quantify the urinary proteins. Among these, Pyrogallol red-molybdate (PRM)[18,19] and Coomassie brilliant blue (CBB)[17] are the most used.[20,21] In human medicine it was shown that different methods for urinary protein quantification yielded discordant results[22,23] and efforts were made to improve agreement.[24,25] Similarly, in dogs, the UPC ratio can be affected by different assays principles and as a consequence dogs with kidney diseases can be incorrectly sub-staged applying the IRIS guidelines. A recent study in dogs showed biases between CBB and PRM in quantification of urinary protein in canine urine and the latter tended to underestimate protein concentration.[26] Moreover, also in cats there are reports demonstrating disagreements between analytical methods different to PRM and CBB.[27,28] Other factors, such as different pre-analytical procedure in different laboratories, storage or pre-dilution have been shown to influence the quantification of urinary protein in dogs.[29,30] On this regard, it's important to highlight that the IRIS guidelines do not specify which method should be used to assess the thresholds proposed in sub-staging feline and canine patients with chronic kidney disease.

No information on the analytical variability of the quantification of urinary protein in cats is available. Therefore, the aims of this study were to determine whether analytic factors affect the evaluation of the UPC ratio in cats. Specifically, the intra-assay and inter-assay repeatability of UPC ratio measurement were evaluated. In addition, agreement between two dye-binding methods (PRM and CBB methods) for measurement of total protein in feline urine was determined.

Materials and Methods

Animals and sample collection

One hundred seventy-four urine samples were prospectively collected from client-owned cats presented for routine diagnostic investigations

Samples were collected from January 2015 to February 2016 at the Veterinary Teaching Hospital (University of Milan) and at a private clinical practice (Veterinary Hospital "Città di Pavia") during routine health screen, under informed consent signed by the owners. According to the ethical committee statements of the University of Milan (number 2/2016), biological samples collected in this setting could be used also for research purposes.

Due to the analytical nature of this study, cats were enrolled irrespective of age, sex and breed or underlining disease and also cats with diseases that could affect urine composition (e.g. CKD, lower urinary tract inflammation, neoplasia, etc.) were included.

Eight to 10 mL of urine were collected from each cat by ultrasonographically-guided cystocentesis. Samples were sent within the syringe to the respective internal clinical pathology laboratories (labeled as "Lab 1" for university of Milan and "Lab 2" for Ospedale Veterinario "Città di Pavia").

Urinalysis

Five millilitres of urine were transferred from the syringe to a sterile conical tube and were macroscopically evaluated for physical properties (color and turbidity) and assayed with dipstick for a semi-quantitative chemical analysis (Combur 10 test, Roche diagnostics, Risch-Rotkreuz, Switzerland). Urine specific gravity (USG) was determined by a handheld refractometer calibrated daily with distilled water (Clinical Refractometer, model 105, Sper Scientific, Scottsdale, AZ, USA).

In order to perform sediment evaluation and supernatant collection, tubes were centrifuged at 450G for 5 minutes (Hermle Z300, Labnet international, Edison, NJ, USA). Then, 4.75 mL of supernatant was removed and transferred in other tubes for subsequent diagnostic biochemical analysis and for study purposes (see below). Supernatants were removed by suction using a dispensable pipette according to current guidelines[31] in order to avoid loss of sediment and supernatant contamination by elements of the sediment. Sediments were resuspended in the remaining 0,25 mL supernatant and slide preparation and microscopic interpretation were performed according to a previous study.[31] Supernatants enrolled in "Lab 1" were used fresh for the analytical procedures described below. Supernatants collected at "Lab 2" were aliquoted (approximately 2 mL each sample) and stored at −20° within 2 hours from collection. Then, aliquots were shipped in batch under controlled temperature to "Lab 1" for inclusion in method comparison study (see below).

Analytical methods

Two commercially available colorimetric test kits were used for protein quantification on urine supernatants in "Lab 1", one based on PRM (Urine proteins, Sentinel diagnostics, Milan, Italy) and the other based on CBB (Total protein Coomassie urine, Far Diagnostics, Pescantina (VR), Italy). The concentration of urinary protein was expressed in mg/dL either for PRM (UP^{PRM}) or for CBB (UP^{CBB}). Both methods were performed according to manufacturer's instructions and were calibrated with the standards provided by the manufacturers. Specifically, PRM standard was stated to be "urinary protein" with no specification of the particular nature of the protein content whereas CBB standard was bovine serum albumin. The protein concentration of the PRM standards provided with the different lots used during the study period ranged from 109 to 122 mg/dL whereas the concentration of CBB standards was 100 mg/dL in all the lots used.
Preliminary assays run in our lab demonstrated that PRM method was linear up to 210 mg/dL as reported by the manufacturer whereas CBB method, independently on the limit of linearity indicated by the producer (400 mg/dL), lose linearity at concentration higher than 120 mg/dL. Therefore, when CBB yielded values higher than 120 mg/dL, supernatants were diluted 1:5 with distilled water; then, samples were re-run with both PRM and CBB and the actual values were calculated based on the dilution factor.

Urinary creatinine concentration (UC) was measured with the modified Jaffe method (Creatinina, Real-Time Diagnostics, Viterbo, Italy) and was expressed as mg/dL. Linearity of the method is up to 30 mg/dL.

When CBB method was applied in a working session, PRM and Jaffe methods were run first, due to the peculiarity of CBB reagent to stain the reagent needle of the automated analyser and the theoretical possibility of contamination and interference of Coomassie dye in the subsequent reaction.

Because urinary creatinine concentration frequently exceeds the range of linearity of the method, supernatants were diluted 1:20 with distilled water in order to measure urinary creatinine and then the actual values were calculated.

Except when differently specified, biochemical tests were performed in triplicate and the mean values were used for data analysis.

All tests were performed with an automated biochemical analyser in Lab1 (Cobas Mira, Roche Diagnostics, Basel, Switzerland) and all methods were daily controlled with QC material (UriChem Level 1 and Level 2, Instrumentation Laboratory, Munich, Germany). Calibration was performed when the Westgard rule 1_{2s} was violated on control solutions.

UPC ratios obtained with PRM (UPC^{PRM}) and, UPC ratios obtained with CBB (UPC^{CBB}) were calculated for each method.

Intra-assay and inter-assay repeatability—The intra-assay imprecision was assessed on twenty fresh urine supernatants, testing samples 20 consecutive times in the same run for protein concentration (with both PRM and CBB methods) and for creatinine concentration; and the UPC ratio was calculated. Mean, SD and CV (calculated as CV = SD/mean X 100) for UP^{PRM}, UP^{CBB}, UC and thus UPC ratio for each method were calculated first on the whole set of samples and then considering separately the results from samples with active (n = 11 samples) and inactive sediment (n = 9 samples).

The inter-assay imprecision was assessed in 15 samples, immediately aliquoted after sampling and stored at -20°C. Each sample was measured on 5 consecutive working days. Urine proteins were measured with both methods (PRM and CBB), urine creatinine was also measured to calculate the UPC ratio with each method. Mean, SD and CV were calculated for UP^{PRM}, UP^{CBB}, UC and thus UPC ratio for each method.

Effect of storage—Since frozen supernatant were used in the method comparison study, a preliminary evaluation of stability at -20°C were performed. To this aim, 25 fresh urinary supernatants were tested immediately after collection (T_0) and after 4 weeks of storage at -20°C (300 µL stored aliquots) with UP^{PRM} and UC after gently thawing and proper mixing before the analysis. This analysis was repeated with further 25 samples testing stability of UP and UPC measured with CBB.

Method comparison study—Forty samples from "Lab1" and 80 samples from "Lab2" were included. Supernatants sent to "Lab1" were analysed fresh within 3 hours from collection, while supernatants from "Lab2" have been stored no longer than 4 weeks at -20°C before the assay.

Urine protein concentration was measured using both PRM and CBB methods, creatinine concentration was measured to allow the calculation of UPC ratios for each method.

UPC ratios obtained with both methods (PRM and CBB) were used to classify the patients as non-proteinuric (NP), borderline proteinuric (BP) or proteinuric (P) according to the IRIS staging system.

Statistical Analysis

A commercially available software (MedCalc® Statistical Software, version 16.8.4, Ostend, Belgium) was used. A P value <0.05 was considered statistically significant. Distribution of variables was assessed by Kolmogorov-Smirnov test.

The possible correlation between intra-assay CV of urinary protein concentration, urinary creatinine concentration or UPC ratio, and the actual values of each of these variables, was investigated with Spearman correlation test. Mann-Whitney *U* test was applied to investigate difference in UP, UC and UPC ratios between samples with active and inactive sediment.

For the evaluation focused on the influence of different storage conditions on UP, UC and UPC ratios, results obtained at T_0 and 1 month later with both PRM and CBB were compared using a Wilcoxon signed rank test.

For the method comparison study, the UP values obtained with PRM and CBB were compared to each other with Wilcoxon signed rank test to assess difference and assayed for correlation with the Spearman test. The same analysis has been run to compare the UPC ratios calculated using the PRM and the CBB method. The agreement between the two methods was assessed by Passing-Bablok and Bland–Altman tests.

The concordance of the two methods in classifying samples according to IRIS staging of proteinuria was assayed with the Cohen's kappa (k) concordance test. The Cohen's k coefficient was used to define concordance as "very good" (k = 0.8–1), "good" (k = 0.6–0.8), moderate (k = 0.4–0.6), "fair" (k = 0.2–0.4), "poor" (k = 0.0–0.2) or "absent" (k <0).[32] Method comparison study tests were performed for the whole set of data and for the sub-sets of samples grouped according to the presence or absence of active sediment

Results

Intra-Assay and inter-assay variability

Descriptive statistics of the samples included in intra-assay and inter-assay evaluation and the respective CVs with regard of UP^{PRM}, UP^{CBB}, UC, UPC^{PRM} and UPC^{CBB} are shown in Table 1. Test for normality revealed a non-Gaussian distribution for both UP, UC and thus for UPC.

The CV was lower for the UC than for UP (and UPC ratio) measured with both PRM and CBB. CBB method appeared more precise than the PRM method. The effect of this variability on sub-staging of sample according to IRIS guidelines was assessed on 4 urine samples that had UPC ratios close to the threshold values (i.e. 0.2 and 0.4) and is shown in Table 2.

No significant differences were found between mean values of UP^{PRM}, UP^{CBB}, UC, UPC^{PRM} and UPC^{CBB} between samples with active and inactive sediment.

No significant correlations were found comparing intra-assay CV and mean values of UP^{PRM} (r = –0.08; P = 0.72), UP^{CBB} (r = –0.29; P = 0.220), UC (r = –0.01; P = 0.95), UPC^{PRM} (r = –0.23; P = 0.33) and UPC^{CBB} (r = –0.19; P = 0.42).

Storage

Compared to T0, UP^{PRM} (median, range: 37.6 mg/dL, 9.2-508.7 mg/dL), UP^{CBB} (median, range: 54.7 mg/dL, 22.5-466.0 mg/dL), UPC^{PRM} (median, range: 0.17, 0.06-6.18) and UPC^{CBB} (median, range: 0.43, 0.06-5.82) did not statistically change after 1 month whereas UC (median, range: 186.7, 64.1-394.7 mg/dL) was significantly higher (P = 0.016).

Method comparison study

Data referred to the whole caseload or to samples with inactive or active sediment are reported in Table 3.

Forty-one (38.7%) urinary samples had an active sediment, while 65 (61.3%) had an inactive sediment. The most common sediment alteration was hematuria (68.3%), followed by leukocyturia (24.4%) and hematuria and leukocyturia (7.3%).

Using PRM, 66, 17 and 37 samples were classified as N, BP and P, respectively, whereas using CBB were 45, 25 and 50, respectively.

CBB yielded constantly higher UP and UPC ratios compared to PRM and the difference was significant (P <0.0001) in all sets of samples.

Urinary protein (PRM: P = 0.0146, CBB: P = 0.0104) and UPC ratio (PRM: P = 0.0035, CBB: P = 0.0087) were significantly different between samples with active and inactive sediment.

Correlations between UP^{PRM} and UP^{CBB}, and between UPC^{PRM} and UPC^{PRM} were highly significant (P <0.0001) in all groups of samples. In the whole set of samples correlation coefficients were 0.82 and 0.91 for urinary proteins and for UPC, respectively; coefficients in the samples with active sediments were 0.96 for both proteinuria and UPC; in the samples with inactive sediments coefficients were 0.78 and 0.96 for protein and for UPC, respectively.

Statistical results of the method comparison study (including intercept and slope with 95% confidence intervals) obtained by Passing-Bablok regression analysis (Figure 1 and Figure 2), and Bland-Altman biases with 95% limits of agreement obtained from UP and UPC ratio in the whole set of sample, in samples with active and with inactive sediments (Figure 3 and Figure 4) were shown in Table 4. Constant and proportional errors were found in all sets of samples, with the exception of UPC in inactive sediment set that yielded no constant bias.

The agreement in staging samples according to IRIS guidelines (Table 4) was defined as “good” in the whole set of samples (k coefficient =0.62), “moderate” for both active and inactive groups of samples (0.59 and 0.56 respectively).

Discussion

In this study, analytical variability in quantification of feline urinary proteins and UPC ratio were evaluated in order to determine their potential effect on clinical decisions. Although from a practical point of view only samples with inactive sediment should be used for UPC interpretation, also samples with active sediment were included in order to highlight the possible analytical difference between the two types of samples.

The two methods for urinary protein quantification yielded CV values similarly to what already found in dogs.[29] A higher value was found with PRM for the sample with protein concentration close to the lower limit of the range of linearity (20 mg/dL) of the method. It's worth to note that the magnitude of CV of this sample could dramatically affect clinical decisions because it could potentially cause shift of the IRIS sub-stage for CKD. However, BP or P samples with low UP and UC are rare (3/120 cases in this study); therefore, the influence of high CVs at low protein concentration is negligible. The CBB method has the advantage to yield on average lower CV values compared to PRM but from a practical standpoint similar numbers of misclassifications were found in samples with UPC close to the two IRIS cut-off. Due to the magnitude of the intra-assay variability, in samples with UPC close to 0.2 and 0.4 it's advisable to interpret results with caution and to repeat measures of UPC over time in order to properly sub-stage feline patients affected by CKD. The inter-assay CVs found in this study were higher than the most common biochemical analytes[33] and could affect clinical decisions even more than intra-assay variability. However, because information about biological variability of proteinuria in cats is not available, it's not known whether these inter-assay CV values could be considered acceptable.

In this study frozen urine samples were used for the method comparison analysis. Although UC statistically increased after one month of storage at -20°C, the lack of statistical differences of UP and UPC ratio after one month of storage at -20°C suggested that measurement of proteinuria may provide reliable results in this setting and confirm that inclusion of frozen samples had no effect on method comparison study. It is important to highlight that the impact of storage on feline urinary samples was not an aim of this study. In human medicine some authors suggested to not use urine samples stored at -20°C for quantification of proteinuria, since fragmentation of proteins (mainly albumin) during storage is described.[21] However, this could be a major problem using immunoassays that detect specific epitopes of albumin. Moreover, protein fragmentation in feline urine needs to be demonstrated and, whether present, it could have affected equally results of both PRM and CBB. Therefore, further evaluations are necessary to better characterize the pre-analytical variability feline urine samples due to different or longer storage conditions.

Among the several commercially available automated methods for measurement of urinary proteins, the two most used dye-binding methods were evaluated in this study. Constant and proportional errors were demonstrated in the whole set of samples and agreement did not improve neither in samples with inactive sediment, where UPC values gain clinical significance.

Similar results have been previously reported in a smaller group of feline samples, comparing different analytical assays (specifically, colorimetric pyrocatechol violet dye-binding says and turbidimetric benzethonium chloride assay).[27] In this study, CBB yielded higher protein concentration and in turn UPC ratios when compared to PRM. Similar positive bias of CBB was demonstrated in dogs for quantification of urinary proteins[26] and total protein in cerebrospinal fluid.[34] Conversely, in human urine CBB tended to yield lower protein concentration when compared to PRM.[24] One important cause of discrepancy between these two methods was shown to be the different responses of dyes to different types of proteins. For example, both methods were shown to constantly underestimate globulin when compared to albumin.[24,35-37] Samples included in this study probably presented a large variability of protein types due to the different underlying diseases and this variability could persist also within the inactive and active sets of samples. This heterogeneity reflected the actual variability of protein patterns in samples commonly assayed in diagnostic laboratories and allowed to quantify analytical variability from a practical point of view. Analysis of the protein content of urine samples was beyond the aim of this study and whether the agreement between methods is different in specific diseases or protein patterns need further research.

Because of the different response to different proteins, the use of the same standard for calibration of different methods and the use of mixed proteins instead of a single protein (such as albumin) as standard solution were proven to improve the agreement between methods.[24] The two methods evaluated in this study were calibrated with the standards provided by the manufactures. The use of the original standards had the aim to evaluate the actual variability that could be found between laboratories. Further studies are needed in order to evaluate whether the agreement between PRM and CBB improves using the same standard, possibly composed by mixed proteins or feline urinary proteins.

The concordance in classifying samples according to the IRIS staging was never in the higher category of classification according to the Cohen's k coefficients (i.e. "very good"). Although concordance in active and inactive subsets of samples was defined moderate and lower than that found in whole set of samples, k coefficients were very close in magnitude and concordance in the three sets of samples could be considered similar. It can be stated that these low concordances were the results of the tendency of CBB to misclassify samples in higher stages, as discussed above. On this regard, it's worth to note that in some cases the magnitude of the bias was so high that samples were graded as non proteinuric with PRM and proteinuric with CBB. These patients would experience different diagnostic approaches and possibly different therapies. Taken together, the results of the method comparison study pointed out that the use of the same laboratory and the same method should be recommended in monitoring patients over time and the comparison of results between different laboratories should be avoided. Moreover, the use of external reference intervals (as determined by IRIS) could worsen the clinical effect of analytical variability. Therefore, according to these results, the use of laboratory specific reference interval, as suggested in human medicine,[23] the modification of the IRIS cut-off relative to the different methods[38] or alternatively the definition of one standard method by IRIS should be advocated.

In conclusion, both methods were precise but samples with UPC close to the cut-off of IRIS substaging should be carefully interpreted to avoid misclassification. Intrinsic difference between analytical methods resulted in inaccuracy and suboptimal concordance in classifying samples according to IRIS substaging. This disagreement could affect clinical decisions, make questionable the comparison of UPC results between different laboratories, and have significant impact in substaging cats affected by CKD, given the strict cut-off recommended in published guidelines in which the method of choice is not indicated.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Tables

Table 1 Precision tests of protein concentration measured with PRM and CBB, creatinine concentration and UPC ratio calculated with both methods. UP, UC and UPC values are described as median and range in brackets; CV values are described as mean ± SD.

	UP^{PRM}		UP^{CBB}		UC		UPC^{PRM}		UPC^{CBB}	
	UP concentration (mg/dL)	CV (%)	UP concentration (mg/dL)	CV (%)	UC concentration (mg/dL)	CV (%)	UPC ratio	CV (%)	UPC ratio	CV (%)
Intra-assay all samples	61.6 (22.8-858.6)	8.4 ±5.2	87.2 (33.4-614.8)	5.6 ±2.6	152.9 (35.3-517.7)	3.4 ±2.5	0.32 (0.05-24.32)	9.5 ±4.8	0.62 (0.15-17.41)	7.2 ±2.6
Intra-assay active sediment	56.5 (22.8-455.6)	9.3 ±6.8	82.8 (43.4-595.0)	5.5 ±2.1	152.8 (70.0-468.5)	3.7 ±2.8	0.32 (0.04-6.6)	10.4 ±6.4	0.61 (0.16-7.06)	7.1 ±2.5
Intra-assay inactive sediment	45.1 (23,9-78.1)	7.9 ±0.8	57.5 (33.4-101.7)	7.3 ±1.4	184.6 (93.9-374.4)	3.8 ±2.4	0.19 (0.16-0.27)	8.2 ±1.1	0.29 (0.15-0.41)	8.3 ±1.9

UP, urinary protein; UP^{PRM}, urinary protein measured with pyrogallol red-molybdate; UP^{CBB}, urinary protein measured with Coomassie brilliant blue; UC, urinary creatinine, UPC, urinary protein-to-creatinine ratio; UPC^{PRM} urinary protein-to-creatinine ratio measured with pyrogallol red-molybdate; UPC^{CBB} urinary protein-to-creatinine ratio measured with coomassie brilliant blue

Table 2 Frequency of misclassification of 4 feline urine with UPC ratios close to IRIS thresholds. When tested with PRM, 2 samples yielded UPC values close to the two IRIS cut-off (0.2 and 0.4). Similarly, two other additional samples yielded UPC values close to the same two cut-off when tested with CBB. Number (and percentage) of shifts of IRIS stage out of the 20 repeated measurements in these samples were countered.

		UPC same stage	UPC different stage
UPC^{PRM}	BP (UPC =0.22)	17 (85%)	3 (15%) NP
	P (UPC =0.42)	13 (65%)	7 (35%) BP
UPC^{CBB}	BP (UPC =0.22)	18 (90%)	2 (10%) NP
	P (UPC =0.41)	11 (55%)	9 (45%) BP

UPC, urinary protein-to-creatinine ratio; UPC^{PRM} urinary protein-to-creatinine ratio measured with pyrogallol red-molybdate; UPC^{CBB} urinary protein-to-creatinine ratio measured with coomassie brilliant blue; BP, borderline proteinuric; P, proteinuric

Table 3: Median (range) of UP, UC and UPC of the 120 samples included in the method comparison. Data of the whole caseload and of samples with inactive or active sediment are shown.

	All samples	Active sediment	Inactive sediment
UP^{PRM} (mg/dL)	28.9 (0.9-919.7)	40.3 (2.3-919.7)[a]	25.5 (0.9-345.3)
UP^{CBB} (mg/dL)	56.6 (2.8-614.8)	74.2 (8.9-595.0)[a]	48.2 (2.8-286.3)
UC (mg/dL)	162.0 (23.9-234.2)	152.9 (23.9-632.6)	158.2 (28.2-520.7)
UPC^{PRM}	0.17 (0.01-24.32)	0.28 (0.02-12.92)[b]	0.15 (0.01-6.97)
UPC^{CBB}	0.31 (0.03-17.41)	0.42 (0.09-14.95)[c]	0.22 (0.03-5.78)

UP^{PRM}, urinary protein measured with pyrogallol red-molybdate; UP^{CBB}, urinary protein measured with Coomassie brilliant blue; UC, urinary creatinine; UPC^{PRM} urinary protein-to-creatinine ratio measured with pyrogallol red-molybdate; UPC^{CBB} urinary protein-to-creatinine ratio measured with coomassie brilliant blue.

Letters indicate which P value refer to comparison between samples with active vs inactive sediment: a <0.05, b P <0.005, c P <0.01

Table 4 Intercept and slope of Passing-Bablok tests and bias and P values recorded in Bland–Altman tests (showed in Figure 2 and 3) of UP and UPC ratios measured with both methods for the whole set of sample and for active and inactive sets of samples. Cohen's k coefficients describing the concordance in classify samples according to International Renal Interest Society (IRIS) are also showed.

		Passing-Bablok		**Bland-Altman**	**Cohen**
		Intercept (95% CI)	**Slope (95% CI)**	**Bias (95% CI)**	**K coefficients**
All	UP	10.70 (6.67 to 14.91)	1.21 (1.10 to 1.33)	-17,82 (-7.50 to -28.14)	
	UPC	0.03 (0.02 to 0.05)	1.27 (1.18 to 1.43)	-0.11 (0.02 to -0.25)	0.62
Active	UP	13.01 (5.27 to 20.77)	1.14 (1.01 to 1.27)	-19.61 (4.72 to -43.95)	
	UPC	0.07 (0.03 to 0.10)	1.15 (1.04 to 1.30)	-0.2 (-0.06 to -0.34)	0.59

Inactive	UP	7.6 (0.39 to 15.44)	1.29 (1.05 to 1.61)	-17.68 (-12.6 to -22.77)	
	UPC	0.01 (-0.02 to 0.04)	1.49 (1.26 to 1.83)	-0.14 (-0.05 to -0.23)	0.56

UP, urinary protein; UPC, urinary protein-to-creatinine ratio

Figure captions

Figure 1 Passing-Bablok plot showing the comparison of urinary protein (UP) between Pyrogallol red-molybdate (PRM) and Coomassie brilliant blue (CBB) obtained from 120 cats in whole set of sample (A) and for active (B) and inactive (C) sets of samples. The blue line is the correlation, the gray line shows best fit and the blue dotted lines represent 95% CI.

Figure 2 Passing-Bablok plot showing the comparison of urinary protein-to-creatinine (UPC) ratio between Pyrogallol red-molybdate (PRM) and Coomassie brilliant blue (CBB) obtained from 120 cats in whole set of sample (A) and for active (B) and inactive (C) sets of samples. The blue line is the correlation, the gray line shows best fit and the blue dotted lines represent 95% CI.

Figure 3 Bland-Altman plot showing the comparison of urinary protein (UP) between Pyrogallol red-molybdate (PRM) and Coomassie brilliant blue (CBB) obtained from 120 cats in whole set (A) of sample and for active (B) and inactive (C) sets of samples. X axes represent the average between the two methods, and the Y axes the indicate the difference between PRM and CBB; the grey line shows the zero bias, the blue solid with the dashed blue lines represent the bias and 95% confidence interval (CI), respectively, the light blue dashed lines are the limits of agreement and the red dotted line is the regression line.

Figure 4 Bland-Altman plot showing the comparison of urinary protein-to-creatine (UPC) ratio between Pyrogallol red-molybdate (PRM) and Coomassie brilliant blue (CBB) obtained from 120 cats in whole set (A) of sample and for active (B) and inactive (C) sets of samples. X axes represent the average between the two methods, and the Y axes the indicate the difference between PRM and CBB; the grey line shows the zero bias, the blue solid with the dashed blue lines represent the bias and 95% confidence interval (CI), respectively, the light blue dashed lines are the limits of agreement and the red dotted line is the regression line.